

# Retinoid Receptor Signaling in Postmitotic Motor Neurons Regulates Rostrocaudal Positional Identity and Axonal Projection Pattern

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## Summary

The identity of motor neurons diverges markedly at different rostrocaudal levels of the spinal cord, but the signals that specify their fate remain poorly defined. We show that retinoid receptor activation in newly generated spinal motor neurons has a crucial role in specifying motor neuron columnar subtypes. Blockade of retinoid receptor signaling in brachial motor neurons inhibits lateral motor column differentiation and converts many of these neurons to thoracic columnar subtypes. Conversely, expression of a constitutively active retinoid receptor derivative impairs the differentiation of thoracic motor neuron columnar subtypes. These findings provide evidence for a regionally restricted role for retinoid signaling in the postmitotic specification of motor neuron columnar identity.

## Introduction

Early in development, neurons acquire identities that permit them to embark on distinct migratory routes, to select defined axonal trajectories, and to form synaptic connections. In the vertebrate central nervous system (CNS), the specification of neuronal identity is initiated by secreted signals provided by local cell groups (Jessell and Melton 1992; Liu and Joyner, 2001). Many of these signals define neuronal fate by regulating the developmental potential of neural progenitor cells through the expression of cell-specific transcription factors (Goridis and Brunet, 1999; Briscoe and Ericson, 2001; Goulding et al., 2002). Less is known, however, about the signaling mechanisms and transcriptional steps involved in the postmitotic diversification of neuronal subtypes.

The link between inductive signaling, transcription factor expression, and neuronal fate has been explored in the spinal cord, most notably in the context of motor neuron specification (Briscoe and Ericson, 2001; Gross

et al., 2002; Shirasaki and Pfaff, 2002). Here, the differentiation of motor neurons requires inductive signals that pattern cells along both the dorsoventral and rostrocaudal axes (Lumsden and Krumlauf, 1996; Briscoe and Ericson, 2001; Persson et al., 2003). Dorsoventral patterning signals, mediated in part by Sonic hedgehog (Shh) secreted from the notochord and floor plate (Patton and Placzek, 2000), impose a specific profile of homeodomain (HD) and basic-helix-loop-helix (bHLH) transcription factor expression in ventral progenitor cells (Briscoe et al., 2000; Briscoe and Ericson, 2001; Muhr et al., 2001; Novitsch et al., 2001; Vallstedt et al., 2001), restricting the differentiation of postmitotic motor neurons to a single progenitor domain (Lee and Pfaff, 2001). Emerging evidence, however, suggests that the diversification of spinal motor neuron subtype occurs only after cell cycle exit (Jessell, 2000; Livet et al., 2002; William et al., 2003).

One major feature of motor neuron diversification is the formation of distinct columnar classes at different rostrocaudal levels of the spinal cord (Landmesser, 1978; Hollyday, 1980a, 1980b; Gutman et al., 1993). At limb levels, many motor neurons acquire a lateral motor column (LMC) identity and extend axons into the limb. Subsequently, LMC neurons generate medial and lateral LMC subtypes, projecting axons to ventrally and dorsally derived limb muscles, respectively (Landmesser, 1978; Hollyday, 1980a, 1980b; Tosney and Landmesser, 1985a, 1985b). In contrast, thoracic levels of the spinal cord generate two other motor neuron columnar subtypes: a set of preganglionic autonomic motor neurons, termed Column of Terni (CT) neurons in the chick, and a set of laterally positioned median motor column (MMC) neurons (Prasad and Hollyday, 1991; Gutman et al., 1993). CT neurons settle in the dorsomedial spinal cord and extend axons to sympathetic targets (Prasad and Hollyday, 1991; Cornbrooks et al., 1997), whereas lateral MMC neurons remain in a ventrolateral position and project axons to body wall muscles (Gutman et al., 1993). The early differentiation of each of these motor neuron subtypes is accompanied by the expression of a distinct LIM HD transcription factor profile (Tsuchida et al., 1994), which in turn regulates motor neuron settling position and axonal projection pattern (Pfaff et al., 1996; Sharma et al., 1998, 2000; Kania et al., 2000; Kania and Jessell, 2003).

The finding that all spinal motor neurons derive from a single dorsoventral domain (Briscoe and Ericson, 2001) implies that signals from the floor plate are unlikely to be involved in directing motor neuron columnar subtype identity. Moreover, transposition of the paraxial mesoderm between brachial and thoracic levels of the neural tube respecifies the rostrocaudal position of generation of LMC neurons (Ensini et al., 1998). Thus, the paraxial mesoderm appears to serve as one source of signals that influence motor neuron columnar fate, although the identity of the relevant mesodermal signals remains unclear.

Several classes of signaling factors are expressed by paraxial mesoderm, including FGFs (Shamim and

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Mason, 1999; Stolte et al., 2002), Wnts (Kawakami et al., 2001), and retinoids (Rossant et al., 1991; Balkan et al., 1992). The synthesis of bioactive retinoids depends on a key synthetic enzyme, retinaldehyde dehydrogenase-2 (Raldh2) (Zhao et al., 1996), which begins to be expressed by paraxial mesodermal cells at the time of overt neural plate formation (Niederreither et al., 1997; Berggren et al., 1999; Swindell et al., 1999; Muhr et al., 1999). An early mesodermal source of retinoids has been implicated in several aspects of progenitor cell differentiation in the caudal neural tube (Muhr et al., 1999; Pierani et al., 1999, 2001; Liu et al., 2001). In particular, retinoid as well as Shh signaling is necessary for the specification of generic spinal motor neuron identity, through activation of expression of the bHLH factor Olig2 (Novitsch et al., 2003 [this issue of *Neuron*]). Strikingly, Raldh2 is later expressed by LMC neurons (Zhao et al., 1996; Sockanathan and Jessell, 1998), and this motor neuronal source of retinoids directs the diversification of LMC neurons into medial and lateral subtypes (Sockanathan and Jessell, 1998).

The involvement of retinoid signaling in many aspects of caudal neural patterning led us to consider whether retinoids might also participate in the specification of motor neuron columnar identity along the rostrocaudal axis of the spinal cord. Two lines of evidence suggest such a role. First, at the time of motor neuron generation, Raldh2 is expressed at a high level by paraxial mesodermal cells that flank brachial (forelimb) levels of the spinal cord, but at a much lower level by paraxial mesoderm at thoracic levels and lumbar levels (see Figures 1A–1C; Niederreither et al., 1997; Berggren et al., 1999; Swindell et al., 1999). Second, transgenic mice that serve as *in vivo* reporters of retinoid signaling have revealed a high level of retinoid signaling activity in the brachial spinal cord but a low level of signaling at thoracic levels (Rossant et al., 1991; Solomin et al., 1998). Collectively, these findings suggest that newly generated brachial motor neurons are exposed to higher levels of retinoid signaling than are thoracic motor neurons. Thus, a differential in retinoid signals provided by the paraxial mesoderm could contribute to the specification of motor neuron columnar identity along the rostrocaudal axis of the spinal cord.

To test this possibility, we have manipulated retinoid receptor signaling in postmitotic spinal motor neurons through expression of dominant-negative or constitutively active retinoid receptor derivatives. Blockade of retinoid receptor signaling in newly generated brachial motor neurons prevents them from acquiring an LMC identity, as assessed by gene expression profile, neuronal settling position, and axonal projection pattern. Moreover, many brachial motor neurons now acquire molecular and anatomical characteristics of thoracic level CT and lateral MMC neurons despite their preserved rostrocaudal position. Conversely, the expression of a constitutively active retinoid receptor derivative in thoracic motor neurons impairs the differentiation of CT and lateral MMC subtypes and leads ultimately to motor neuron death. Thus, the status of retinoid receptor signaling in postmitotic motor neurons appears to regulate motor neuron columnar subtype identity along the rostrocaudal axis of the spinal cord.

## Results

### Manipulation of Retinoid Receptor Signaling in Postmitotic Motor Neurons

To manipulate retinoid signaling in spinal motor neurons, we expressed retinoid receptor derivatives that act in either a dominant-negative or constitutively active manner. To inhibit retinoid receptor signaling, we expressed a RAR $\alpha$  derivative, RAR403, that lacks the AF2 ligand-dependent transactivation domain (Damm et al., 1993; see Supplemental Figure S1 at <http://www.neuron.org/cgi/content/full/40/1/97/DC1>). To activate retinoid receptor signaling constitutively, we expressed a human RAR $\alpha$  fusion with the transcriptional activator VP16 (Blumberg et al., 1997; Lipkin et al., 1996; Supplemental Figure S1). Both RAR derivatives were expressed in a bicistronic IRES-based vector (Arber et al., 1999), in which the first cistron encodes the retinoid receptor and the second a nuclear targeted  $\beta$ -galactosidase protein (NLZ) (to localize neuronal nuclei) or enhanced green fluorescent protein (eGFP) (to visualize neurons in conjunction with HRP labeling) (Supplemental Figure S1).

To direct transgene expression to postmitotic motor neurons, retinoid receptor derivatives were expressed under the control of a 9 kb 5' flank region of the mouse *Hb9* gene that excludes expression from spinal progenitor cells (Arber et al., 1999; Sharma et al., 2000; William et al., 2003). We detected >95% coincidence in neuronal expression of genes encoded by the first and second cistrons (data not shown), and typically >25% of motor neurons expressed the transgene. The differentiation of motor neurons after expression of dominant-negative or constitutively active retinoid receptors was compared with that of motor neurons expressing LacZ or eGFP plasmids under *Hb9* control.

### Expression of RAR403 Blocks the Specification of LMC Identity

We initially examined the consequences of disrupting retinoid receptor signaling in motor neurons at brachial levels of the spinal cord. The neural tube of chick embryos was electroporated at stages 12 to 14 with NLZ, eGFP, RAR403.NLZ, or RAR403.eGFP constructs, and embryos were permitted to develop until stages 23 to 32 for analysis. The expression of these constructs did not compromise general aspects of motor neuron differentiation, as revealed by the persistence of expression of the motor neuron transcription factors Isl1/2 and HB9 (Figures 1D and 1H; data not shown).

Since retinoid signaling has been implicated in the specification of lateral LMC identity (Sockanathan and Jessell, 1998; Solomin et al., 1998), we tested the efficacy of RAR403 as an inhibitor of retinoid signaling by examining the differentiation of lateral LMC neurons, marked by coexpression of Isl2 and Lim1. After expression of a control NLZ construct and analysis at stages 23 and 27, ~30% of LacZ<sup>+</sup> LMC neurons coexpressed Lim1 (Figures 1D–1G, 1L, and 1M), and there was no change in the total number of Isl2<sup>+</sup>, Lim1<sup>+</sup> lateral LMC motor neurons on the electroporated side of the spinal cord (Figures 1D–1K). After expression of RAR403.NLZ, there was no change in the total number of motor neurons, but fewer than 2% of LacZ<sup>+</sup> LMC neurons coex-

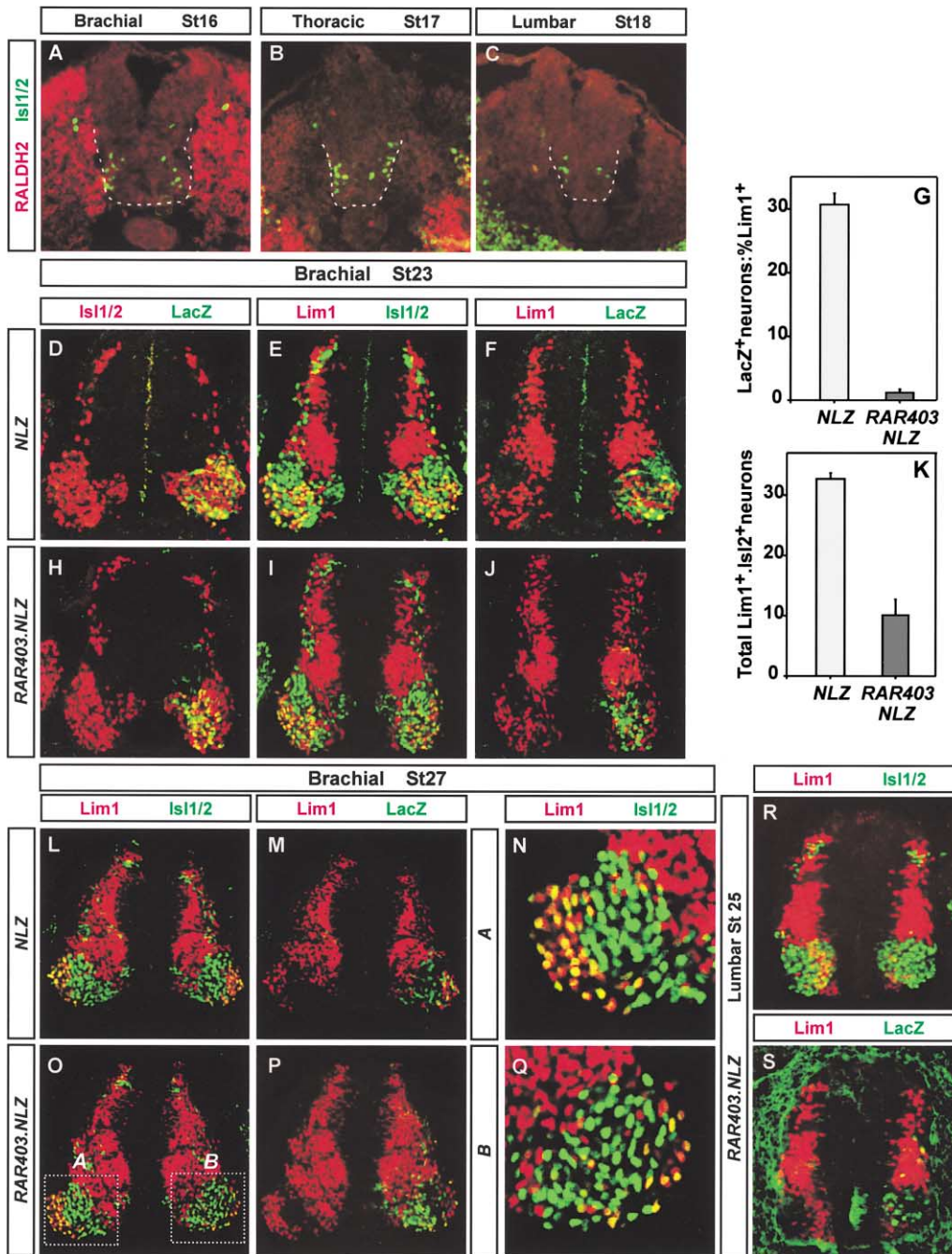


Figure 1. Blockade of Retinoid Signaling Results in the Loss of Lateral LMC Neurons

(A–C) Raldh2 (red) is expressed in paraxial mesoderm at brachial (A) but not thoracic (B) or lumbar (C) levels of the spinal cord at the onset of motor neuron generation. Postmitotic motor neurons are visualized by Isl1/2 expression (green).

(D–F and H–J) Analysis of stage 23 embryos electroporated with *NLZ* (D–F) or *RAR403.NLZ* (H–J). In all panels, the electroporated side is on the right.

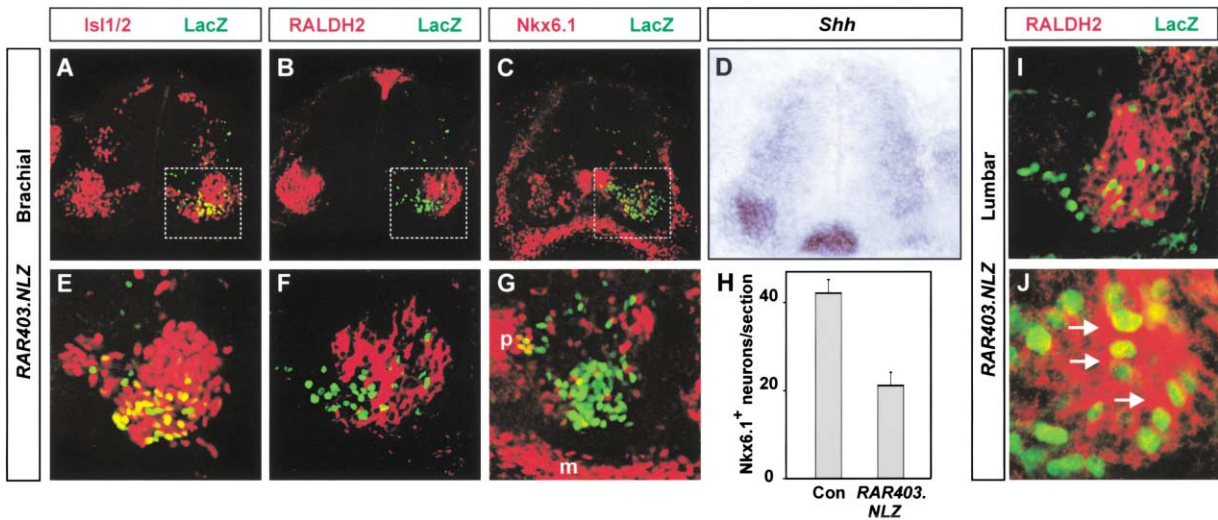
(G) Percentage of LacZ<sup>+</sup> neurons that express Lim1 when electroporated with *NLZ* or *RAR403.NLZ*.

(K) Total number of Lim1<sup>+</sup>, Isl2<sup>+</sup> lateral LMC neurons in embryos electroporated with *NLZ* or *RAR403.NLZ*. See Experimental Procedures for quantification. Differences in (G) and (K) are significant ( $p < 0.0001$ ;  $p = 0.000002$ , respectively). Total motor neuron numbers: control side  $182 \pm 8$  neurons/section; electroporated side  $183 \pm 11$  neurons/section.

(L–Q) Analysis of stage 27 embryos electroporated with *NLZ* (L and M) and *RAR403.NLZ* (O and P). High-power images of the areas boxed in (O) (A and B) are shown in (N) and (Q), respectively.

(R and S) LMC differentiation in lumbar spinal cord electroporated with *RAR403.NLZ* and analyzed at stage 25.





**Figure 2. Blockade of Retinoid Signaling Results in the Loss of LMC Identity**

In all panels the electroporated side of the embryo is to the right.

(A–D) Embryos electroporated with *RAR403.NLZ* were analyzed at stage 27 for expression of *RALDH2* (B), *Nkx6.1* (C), and *Shh* (D).

(E–G) High-power images of the boxed areas marked in (A)–(C). The coexpression of *LacZ* in medially located *Nkx6.1*<sup>+</sup> cells corresponds to expression in progenitor cells (p); expression of *Nkx6.1* is also seen in adjacent mesoderm (m).

(H) Number of *Nkx6.1*<sup>+</sup> motor neurons on the electroporated (*RAR403.NLZ*) and controlateral (Con) sides of experimental embryos. This difference is significant ( $p = 0.00024$ ).

(I and J) Lumbar level spinal cord electroporated with *RAR403.NLZ*, analyzed at stage 26. Arrows in (J) mark examples of *LacZ*<sup>+</sup> neurons that express *Raldh2*.

pressed *Lim1* (Figures 1G–1K and 1N–1Q), and there was a >3-fold reduction in *Isl2*<sup>+</sup>, *Lim1*<sup>+</sup> neurons (Figure 1K). Thus, expression of a dominant-negative retinoid receptor blocks the specification of lateral LMC identity, extending findings that the differentiation of this class of motor neurons requires exposure to retinoids (Sockanathan and Jessell, 1998).

The expression of a high level of *Raldh2* in brachial paraxial mesoderm (Figures 1A–1C) led us to examine whether dominant-negative retinoid receptor expression might also exert an earlier influence on brachial LMC specification. To test this possibility, we expressed *NLZ* and *RAR403.NLZ* in brachial motor neurons and assayed three markers of LMC neurons: *Raldh2* (Sockanathan and Jessell, 1998), *Shh* (A. Kottmann and T.M.J., unpublished observations), and *Nkx6.1* (Cai et al., 2000; data not shown). Expression of *NLZ* in brachial motor neurons did not affect expression of *Raldh2*, *Nkx6.1*, or *Shh* (data not shown), but expression of *RAR403.NLZ* resulted in a cell-autonomous extinction in *Raldh2* (Figures 2A, 2B, 2E, and 2F) and *Nkx6.1* (Figures 2A, 2C, 2E, 2G, and 2H) expression and blocked *Shh* expression (Figure 2D). Thus, blockade of retinoid receptor signaling in brachial motor neurons appears to block the acquisition of generic as well as lateral LMC character.

We next addressed the source of retinoids involved in the specification of brachial LMC character. The initial specification of brachial LMC identity could be imposed by retinoids derived from brachial paraxial mesoderm. Alternatively, a nonretinoid signal from the paraxial mesoderm could establish an initial LMC character that is then stabilized by retinoids provided by LMC neurons themselves. To distinguish these possibilities, we took advantage of the observation that at the time of specifi-

cation of lumbar LMC identity, surrounding lumbar level paraxial mesoderm expresses only a very low level of *Raldh2* (Figure 1C; Hollyday and Hamburger, 1977; Berggren et al., 1999; Swindell et al., 1999). We reasoned, therefore, that if mesodermal retinoids initiate LMC differentiation at brachial levels, then *RAR403* expression would be expected to block brachial but not lumbar LMC differentiation. In contrast, if retinoid signals from motor neurons are involved in LMC specification, then *RAR403* expression would be expected to block LMC differentiation at lumbar as well as brachial levels.

We therefore expressed *RAR403.NLZ* at lumbar levels and examined generic LMC differentiation, assessed by *Raldh2* expression. Expression of *RAR403.NLZ* in lumbar level motor neurons failed to extinguish *Raldh2* expression (Figures 2I and 2J), in marked contrast to our findings at brachial levels. The differentiation of *Isl2*<sup>+</sup>, *Lim1*<sup>+</sup> lateral LMC neurons was, however, blocked by *RAR403.NLZ* expression at lumbar as well as at brachial levels (Figures 1R and 1S). These findings argue that retinoid signals from LMC neurons themselves are not involved in the consolidation of early LMC identity. It follows, therefore, that the loss of LMC differentiation observed after dominant-negative retinoid receptor expression at brachial levels does reflect the blockade of retinoid signaling from brachial paraxial mesoderm.

#### Blockade of Retinoid Signaling in Brachial Motor Neurons Alters Cell Position and Axonal Trajectory

We next explored the influence of retinoids on other aspects of LMC differentiation. At brachial levels, the ventrolateral positioning of LMC neurons becomes evident by stages 26 to 27 (Hollyday and Hamburger, 1977;

Sockanathan and Jessell, 1998). We therefore examined whether the loss of LMC specification after expression of *RAR403.NLZ* influences the settling pattern of brachial motor neurons. To assign the position of LacZ<sup>+</sup> motor neurons in embryos electroporated with *NLZ* or *RAR403.NLZ*, we monitored motor neuron settling within a grid coordinate (Figure 3; see Experimental Procedures). After expression of *NLZ*, LacZ<sup>+</sup> motor neurons were distributed throughout the entire motor neuron settling domain (Figures 3A and 3B). In contrast, after *RAR403.NLZ* expression, most LacZ<sup>+</sup> neurons were clustered in a ventromedial position and were almost completely excluded from the normal settling position of LMC neurons (Figures 3C and 3D;  $p < 0.005$  versus *NLZ* controls). Thus, blockade of retinoid receptor activation in postmitotic brachial motor neurons prevents them from acquiring the lateral settling position characteristic of LMC neurons.

We also examined whether blockade of retinoid receptor signaling prevents brachial motor neurons from projecting their axons out of the spinal cord and into the developing limb, a later defining feature of LMC identity. Injection of FITC-dextran into the ventral roots of *RAR403.eGFP*-electroporated embryos labeled many GFP<sup>+</sup> motor neurons (Figures 3E–3G), indicating that brachial motor neurons can extend axons after blockade of retinoid receptor signaling. To assess the efficiency with which motor axons projected into the limb, horseradish peroxidase (HRP) was injected at stage 29 into the forelimb of *NLZ*-, *eGFP*-, *RAR403.eGFP*-, or *RAR403.NLZ*-electroporated embryos, and the position and marker status of retrogradely labeled motor neurons was examined. After HRP injection into *NLZ*- or *eGFP*-electroporated embryos, ~70% of LacZ<sup>+</sup> or eGFP<sup>+</sup>, Isl1/2<sup>+</sup> motor neurons were retrogradely labeled with HRP (Figures 3K and 3L; data not shown). In contrast, in embryos electroporated with *RAR403.eGFP* or *RAR403.NLZ*, only ~10% of Isl1/2<sup>+</sup>, LacZ<sup>+</sup> motor neurons labeled with HRP (Figures 3H, 3J, and 3K). Thus, blockade of retinoid receptor signaling in brachial motor neurons reduces, but does not completely inhibit (see also Supplemental Figure S2 at <http://www.neuron.org/cgi/content/full/40/1/97/DC1>), the projection of motor axons into the limb. The finding that motor neurons can project axons into the limb in the absence of an LMC character is consistent with studies showing that thoracic motor neurons project axons into the limb if grafted to limb levels of the spinal cord (O'Brien et al., 1990; O'Brien and Oppenheim, 1990).

#### Brachial Motor Neurons that Express *RAR403* Do Not Acquire Medial MMC Character

What is the fate of brachial motor neurons after blockade of retinoid receptor signaling? Brachial levels of the spinal cord generate medial MMC as well as LMC neurons (Landmesser, 1978; Gutman et al., 1993; Tsuchida et al., 1994), raising the possibility that the blockade of retinoid receptor activation converts prospective LMC neurons to medial MMC neurons. To address this issue, we examined whether brachial motor neurons that express *RAR403.NLZ* coexpress Lim3, a defining molecular marker of medial MMC identity (Tsuchida et al., 1994; Sharma et al., 1998). The proportion of Isl1/2<sup>+</sup>, LacZ<sup>+</sup>

motor neurons that expressed Lim3 was similar in *NLZ*- and *RAR403.NLZ*-electroporated embryos (Figures 4A–4G), indicating that inhibition of retinoid receptor signaling in brachial motor neurons does not promote the differentiation of medial MMC neurons, at least as assessed by LIM HD profile.

To explore whether other aspects of medial MMC character are acquired after blockade of retinoid receptor signaling, we examined whether brachial motor neurons show an increase in the incidence of axonal projections to axial muscles, a trajectory characteristic of medial MMC neurons (Tosney and Landmesser, 1985a, 1985b; Gutman et al., 1993). To test this, HRP was injected at stage 29 into the axial musculature of *RAR403.eGFP*-electroporated embryos. About 20% of the total population of HRP-labeled eGFP<sup>+</sup> motor neurons expressed Lim3 (Figures 4H–4J), close to the normal fraction of brachial motor neurons with medial MMC identity (Sockanathan and Jessell, 1998). Thus, the inhibition of retinoid receptor activation appears to block the specification of LMC identity without promoting a medial MMC-like identity.

#### Prospective Brachial LMC Neurons Acquire Thoracic Columnar Characters after Blockade of Retinoid Receptor Signaling

As motor neurons are generated, the level of retinoid signaling from paraxial mesoderm flanking thoracic levels of the spinal cord is much lower than that at brachial levels (Figures 1A–1C; Berggren et al., 1999; Swindell et al., 1999; Solomin et al., 1998). This observation led us to consider whether brachial motor neurons in which retinoid receptor signaling has been blocked acquire characteristics of CT and lateral MMC neurons (Prasad and Hollyday, 1991; Gutman et al., 1993).

CT neurons have a distinctive migratory pattern, settling in the dorsomedial spinal cord by stage 29 (Figure 5A; Prasad and Hollyday 1991; Cornbrooks et al., 1997). They also exhibit a distinctive profile of HD protein expression: initially they coexpress Isl and HB9 proteins but rapidly downregulate HB9 expression prior to their dorsomedial migration (Figures 5A and 5D; Tsuchida et al., 1994; J.P. Thaler et al., submitted; William et al., 2003). We examined whether brachial motor neurons acquire these CT characteristics after blockade of retinoid receptor activation. After brachial electroporation of *RAR403.NLZ* and analysis at stage 29, ~30% of LacZ<sup>+</sup> neurons were found in a dorsomedial position characteristic of CT neurons (Figures 5B, 5C, and 5G), whereas no dorsomedially located motor neurons were detected in *NLZ*-electroporated embryos (Figure 5G, data not shown). Many of the ectopic motor neurons in *RAR403.NLZ* electroporated embryos expressed Isl proteins (Figures 5B and 5C), and most had extinguished HB9 expression (Figures 5E and 5F). Thus, some brachial motor neurons that express *RAR403.NLZ* acquire the migratory behavior and HD profile of CT neurons.

CT neurons project their axons to sympathetic targets (Prasad and Hollyday, 1991), so we examined whether the CT-like neurons generated at brachial levels after *RAR403* expression innervated sympathetic chain ganglia. Injection of HRP into sympathetic chain ganglia at brachial, T1, and T2 segmental levels of *RAR403.eGFP*-

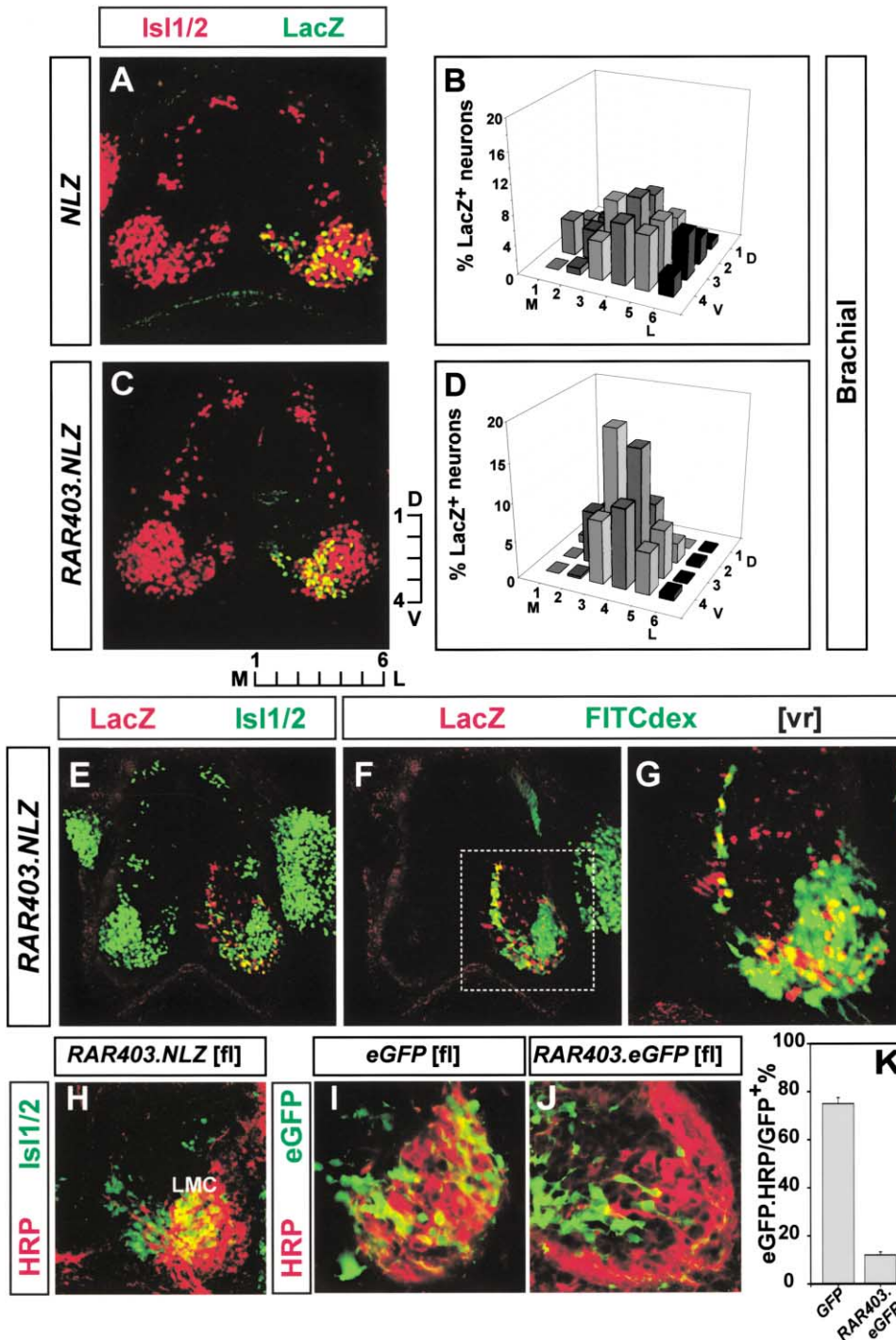


Figure 3. Brachial Motor Neurons that Express *RAR403* Occupy a Medial Position and Show Reduced Projections to the Limb

(A and B) Neurons electroporated with *NLZ* are distributed throughout the LMC at stage 27. (C and D) Neurons electroporated with *RAR403.NLZ* occupy a more ventromedial position. (E–G) Motor neuron labeling after FITC-dextran injections into the ventral root ([vr]) of *RAR403.NLZ* electroporated embryos. (G) High-power images of the boxed area in (F) show many LacZ<sup>+</sup> neurons labeled with FITC-dextran. (H–K) Motor neuron labeling after HRP injection into forelimb ([fl]) of (H) *RAR403.NLZ*-, (I) *eGFP*-, or (J) *RAR403.eGFP*-electroporated embryos. (K) Percentage of eGFP<sup>+</sup> neurons colabeled with HRP in *eGFP* or *RAR403.eGFP* embryos ( $p < 0.00001$ ).

electroporated embryos labeled dorsomedially located brachial eGFP<sup>+</sup> motor neurons (Figures 6C–6F; typically ~2–3 eGFP<sup>+</sup>, HRP-labeled motor neurons were de-

tected per 10  $\mu$ m section). In contrast, no HRP-labeled dorsomedial motor neurons were detected at brachial levels after similar HRP injections in wild-type embryos

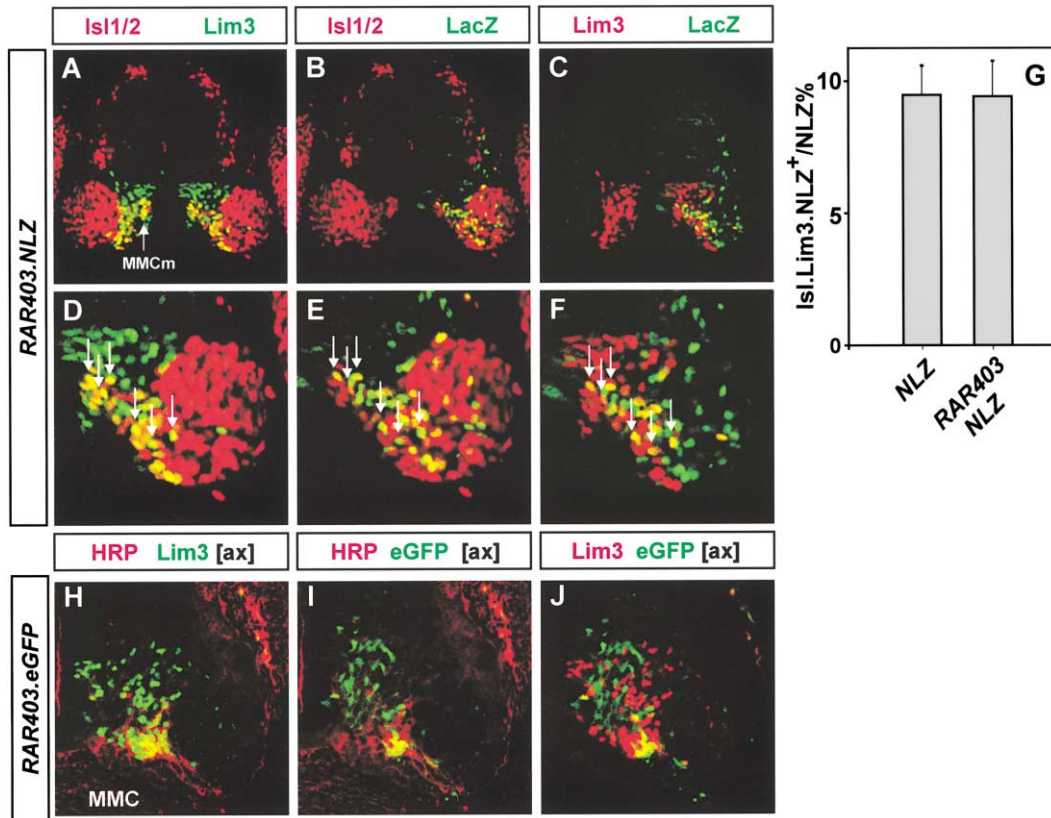


Figure 4. Blockade of Retinoid Signaling in Brachial Motor Neurons Does Not Affect Medial MMC Identity

(A–F) Analysis of medial MMC identity in *RAR403.NLZ* expressing motor neurons at stage 27, as assessed by Lim3 and Isl coexpression. Arrows mark motor neurons that express *RAR403.NLZ*, Isl1/2, and Lim3.

(G) Percentage of LacZ<sup>+</sup> neurons that express Isl and Lim3 proteins in *NLZ*- and *RAR403.NLZ*-electroporated embryos. No significant difference is observed in the number of Lim3<sup>+</sup> motor neurons ( $p = 0.96$ ).

(H–J). Retrograde labeling of medial MMC neurons after injection of HRP into axial muscles ([ax]) of *RAR403.eGFP*-electroporated embryos at stage 29. Some Lim3<sup>+</sup>, eGFP<sup>+</sup> neurons were HRP labeled; however, the majority of eGFP<sup>+</sup> neurons are not retrogradely labeled (I and J).

(Figures 6A and 6B). Thus, some brachial motor neurons that express *RAR403.eGFP* project their axons to sympathetic chain ganglia, a feature of CT neurons.

As a further means of assessing the targets of brachial motor neurons that express *RAR403.eGFP*, we traced eGFP-labeled motor axons that emerged from brachial ventral roots. In embryos electroporated with eGFP alone, few if any eGFP<sup>+</sup> axons were found within sympathetic chain ganglia at brachial levels (Figures 6H and 6L). In contrast, in *RAR403.eGFP*-electroporated embryos, many eGFP<sup>+</sup> axons branched from the peripheral nerve into the core of brachial sympathetic chain ganglia (Figures 6I, 6J, 6M, and 6N). Together, these findings provide evidence that blockade of retinoid receptor signaling converts some prospective brachial LMC neurons into CT-like motor neurons.

Only a minority of prospective brachial LMC neurons acquire CT-like properties after blockade of retinoid receptor signaling, however, raising the possibility that some motor neurons instead acquire a lateral MMC character. Lateral MMC neurons settle in the ventrolateral region of the thoracic spinal cord (Gutman et al., 1993; Tsuchida et al., 1994) and can be distinguished from LMC and medial MMC neurons by expression of Isl1/2 in the absence of Raldh2 or Lim3 (Tsuchida et al.,

1994; Sockanathan and Jessell, 1998; Sharma et al., 1998). After electroporation of *RAR403.NLZ* at brachial levels, ~70% of LacZ<sup>+</sup>, Isl1/2<sup>+</sup> motor neurons remained in a ventrolateral position (Figures 5C and 5G), and ~60% of these LacZ<sup>+</sup> neurons lacked Lim3 and Raldh2 expression (Figures 5C and 5G; data not shown), consistent with a lateral MMC identity.

Lateral MMC neurons also differ from CT and LMC neurons in their innervation pattern, projecting axons to the intercostal muscles of the body wall (Gutman et al., 1993). Injection of HRP into intercostal muscles of *RAR403.eGFP*-electroporated embryos revealed many ventrolateral HRP-labeled Isl1/2<sup>+</sup>, Lim3<sup>−</sup> brachial motor neurons (Figures 7D–7G). In contrast, no HRP-labeled brachial motor neurons were detected after similar HRP injections in wild-type embryos (Figures 7A–7C). Together, these findings support the idea that blockade of retinoid receptor activation in prospective brachial LMC neurons also results in the differentiation of motor neurons with lateral MMC-like character.

To control for the specificity of *RAR403* function, we examined whether the differentiation of thoracic motor neurons is affected by blockade of retinoid receptor signaling. After thoracic expression of *RAR403.NLZ*, no change in the differentiation of medial MMC, lateral



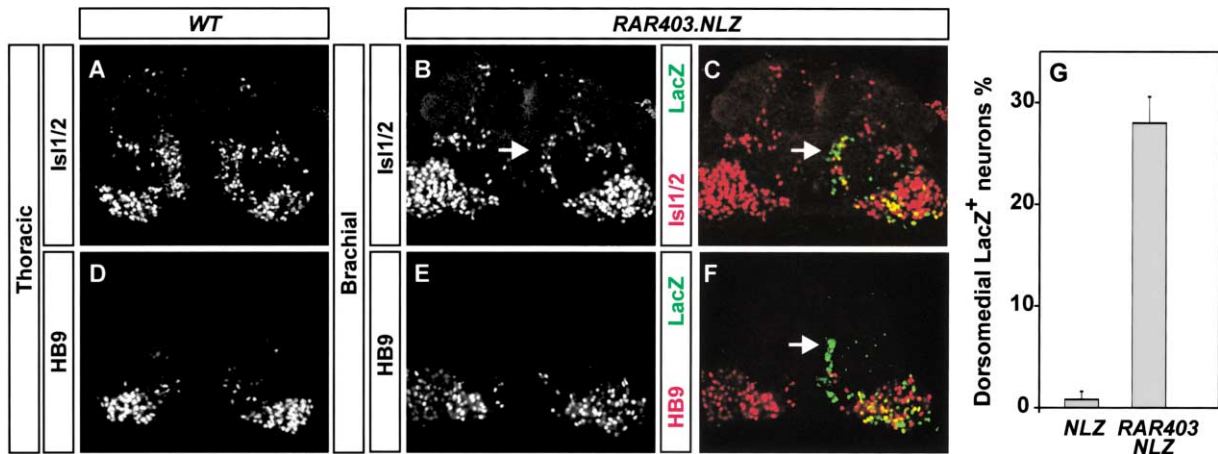


Figure 5. Some Brachial Motor Neurons that Express RAR403 Exhibit a CT-like Character

(A and D) At thoracic levels of wild-type (wt) embryos, CT neurons are located dorsomedially and express *Isl1/2* (A) but little or no *HB9* (D). (B, C, E, and F) Brachial levels of stage 29 embryos electroporated with *RAR403.NLZ*. Arrows mark dorsomedially located *RAR403.NLZ*<sup>+</sup> neurons. (G) Percentage of *LacZ*<sup>+</sup> neurons located in a dorsomedial position in *NLZ*- and *RAR403.NLZ*-electroporated embryos. The difference in motor neuron number is significant ( $p < 0.001$ ).

MMC, or CT neurons was detected, as assessed by LIM HD protein profile and neuronal settling position (Supplemental Figure S3 at <http://www.neuron.org/cgi/content/full/40/1/97/DC1>). Thus, retinoid receptor activation in newly generated thoracic motor neurons does not seem to be required for the specification of CT or lateral MMC identity.

#### Expression of a Constitutively Active Retinoid Receptor at Thoracic Levels Perturbs CT and Lateral MMC Differentiation

Since inhibition of retinoid receptor signaling in brachial motor neurons blocks the specification of LMC fate and promotes CT and lateral MMC character, we considered whether the activation of retinoid receptor signaling at thoracic levels might, conversely, promote LMC and repress CT and lateral MMC columnar identities. We examined this issue by monitoring motor neuron columnar fates in thoracic spinal cord after expression of *VP16RAR*, a retinoid receptor derivative that activates retinoid receptor signaling in a constitutive, ligand-independent, manner (Lipkin et al., 1996). Expression of *VP16RAR.NLZ* in thoracic motor neurons did not impair the specification of medial MMC identity, assessed by the number of *LacZ*<sup>+</sup> motor neurons that coexpressed *Lim3* at stage 25 and stage 28 (Figure 8A; data not shown). In addition, the LMC marker *Raldh2* was not induced in thoracic *LacZ*<sup>+</sup>, *Isl*<sup>+</sup> motor neurons (Figure 8B). Thus, retinoid receptor activation in thoracic level motor neurons does not induce an LMC-like character.

In contrast, expression of *VP16RAR.NLZ* at thoracic levels of the spinal cord disrupted CT and lateral MMC differentiation. The early downregulation of *HB9* expression characteristic of CT neurons occurred on schedule at stage 22–23 (Figures 8C–8E; William et al., 2003), but by stage 29 we detected a marked depletion of dorsomedially positioned *Isl*<sup>+</sup> CT neurons (Figures 8F and 8G). In addition, expression of *VP16RAR.NLZ* significantly decreased the number of ventrally positioned

*Isl1/2*<sup>+</sup>, *Lim3*<sup>−</sup> lateral MMC-like motor neurons compared to *NLZ* controls (Figure 8H;  $p < 0.005$  versus controls). These findings provide evidence that constitutive retinoid receptor activation perturbs the differentiation of both CT and lateral MMC neurons.

What is the basis of the defect in CT and lateral MMC differentiation? From stage 25 onward, thoracic expression of *VP16RAR.NLZ* resulted in a reduction in the number of *LacZ*<sup>+</sup> motor neurons (Figure 8F), and most of the residual *LacZ*<sup>+</sup> neurons were found in the medial MMC (Figure 8F, and data not shown). We therefore examined whether thoracic motor neurons die under conditions of *VP16RAR.NLZ* expression, assaying the number of ventral TUNEL<sup>+</sup> nuclei in the thoracic spinal cord at stages 23 and 25. A marked increase in TUNEL<sup>+</sup> nuclei was detected at both stages, compared to controls (Figure 8I). Thus, expression of *VP16RAR.NLZ* in thoracic motor neurons leads to the apoptotic death of many prospective CT and lateral MMC neurons.

We were concerned that *VP16RAR.NLZ* expression might simply be toxic to many spinal motor neurons. To examine this possibility, we expressed *VP16RAR.NLZ* in brachial motor neurons, an axial level where retinoid receptor signaling is apparently involved in LMC specification. Brachial expression of *VP16RAR.NLZ* did not affect the differentiation of LMC or medial MMC neurons or markedly affect motor neuron number (Figures 8J–8M; data not shown). The findings that *VP16RAR.NLZ* expression is without effect on the generation of medial MMC neurons and does not influence LMC differentiation, therefore, argue strongly against a nonspecific toxic action, revealing a selective sensitivity of thoracic columnar subtypes to *VP16RAR* expression. These data support the idea that postmitotic retinoid receptor activation inhibits the normal progression of thoracic level motor neurons to CT and lateral MMC columnar identities.

These findings, therefore, complement observations at brachial levels, where retinoid receptor activation appears to be required for the specification of LMC fate.



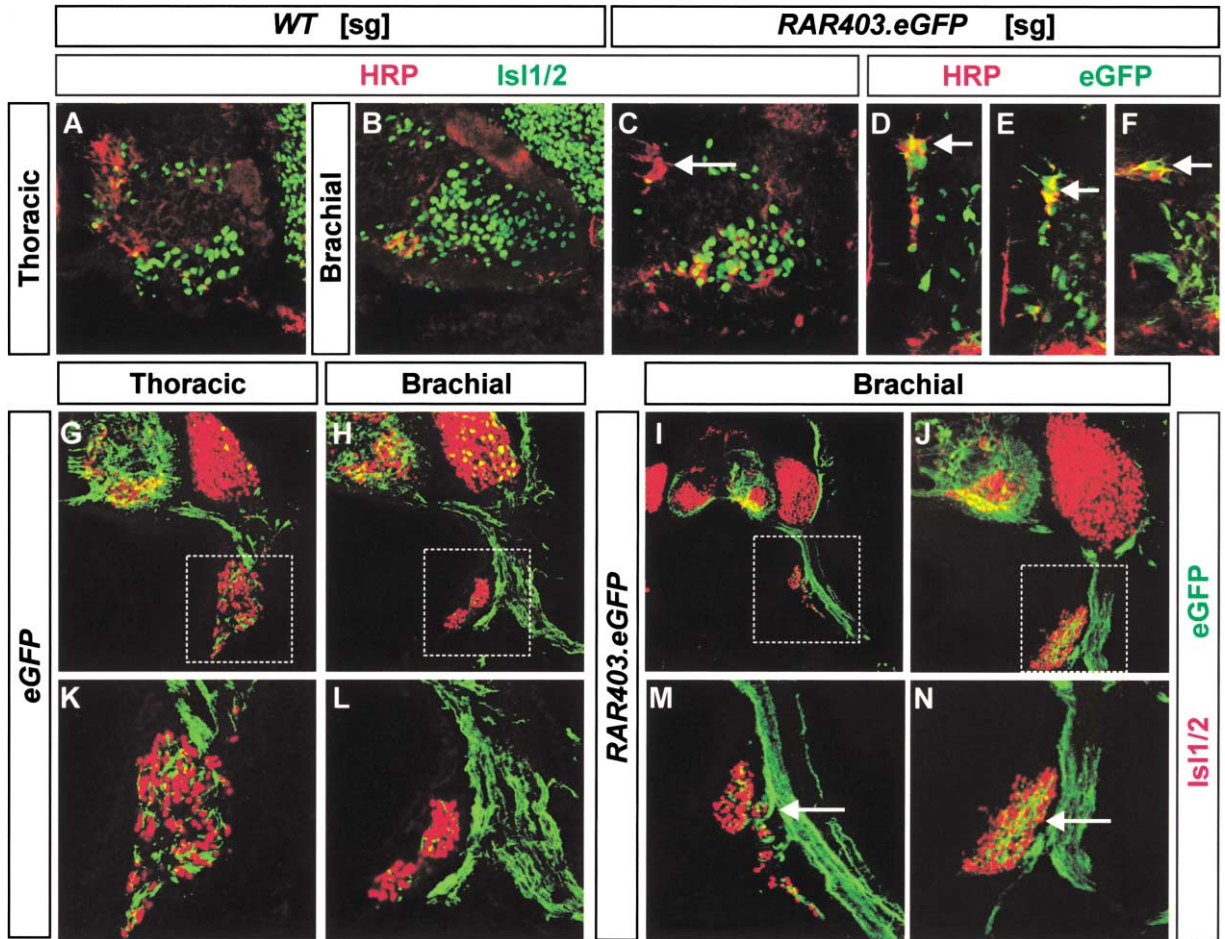


Figure 6. Some Brachial Motor Neurons that Express RAR403 Project to Sympathetic Chain Ganglia

(A and B) Injection of HRP into sympathetic chain ganglia ([sg]) retrogradely labels CT neurons at thoracic regions (A) and a ventromedial cluster (B) of motor neurons at brachial levels.

(C–F) Injection of HRP in chain sympathetic ganglia of *RAR403.eGFP*-electroporated embryos retrogradely labels dorsomedial GFP<sup>+</sup> neurons with HRP (arrows). (D–F) High-power images of dorsomedially located HRP-labeled eGFP<sup>+</sup> neurons.

(G–N) Orthograde eGFP labeling of motor axons after electroporation with either eGFP (G, H, K, and L) or *RAR403.eGFP* (I, J, M, and N). Panels (K), (L), (M), and (N) are close-ups of the boxed areas in the corresponding upper panels. Arrows mark (M) axonal projections to and (N) axonal ramification within sympathetic ganglia in *RAR403.eGFP*-expressing brachial motor neurons.

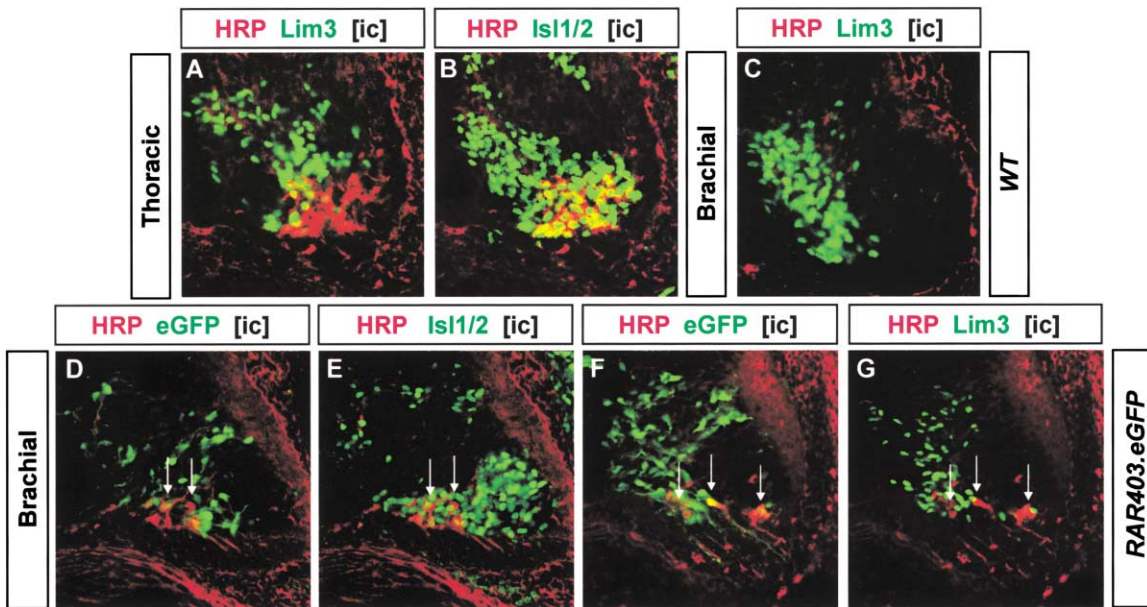
Thus, the status of retinoid receptor activation in newly generated motor neurons seems to be a critical determinant of the differentiation of motor neuron columnar subtypes formed at brachial and thoracic levels of the spinal cord.

## Discussion

The allocation of motor neurons to distinct columnar subtypes is an early step in the formation of specific motor projection patterns. We show here that the status of retinoid receptor activation in postmitotic spinal motor neurons is a critical step in the specification of their columnar identity. In particular, retinoid receptor signaling appears necessary for the specification of LMC identity at brachial levels of the spinal cord. We discuss below how spatial restrictions in retinoid signaling contribute to motor neuron columnar diversification and how retinoid signaling is integrated with other patterning events that specify motor neuron subtype fate.

## Retinoid Receptor Signaling in Postmitotic Motor Neurons Is Required for Brachial LMC Identity

One major conclusion of these studies is that the specification of LMC identity at brachial levels of the spinal cord depends on retinoid receptor activation in postmitotic motor neurons. Together with other recent data, these findings reveal that retinoid signaling has a profound impact on the progressive differentiation of LMC neurons. The exposure of progenitor cells in the ventral neural tube to low levels of retinoids derived from the nascent paraxial mesoderm is required for the activation of Pax6 and Olig2 expression (Novitsch et al., 2003 [this issue of *Neuron*]). In turn, these two transcription factors direct ventral progenitor cells to a generic motor neuron fate (Ericson et al., 1997; Novitsch et al., 2001; Lu et al., 2002; Zhou and Anderson, 2002). Our data suggest that a later phase of retinoid receptor activation in postmitotic motor neurons is an essential step in the emergence of a brachial LMC identity. The differentiation of brachial LMC neurons is, however, accompanied by the selective



**Figure 7. Some Brachial Motor Neurons that Express RAR403 Innervate Body Wall Muscles**  
Retrograde labeling of brachial motor neurons after injection of HRP into intercostal muscles ([ic]) of stage 29 embryos. (A–C) In wild-type embryos (wt), many ventrolateral Lim3<sup>+</sup>, HRP<sup>+</sup> neurons are detected at thoracic levels (A and B), but no Lim3<sup>+</sup>, HRP<sup>+</sup> neurons are detected at limb levels (C). (D–G) In *RAR403.eGFP*-electroporated embryos, Lim3<sup>+</sup>, HRP<sup>+</sup> neurons are detected in ventrolateral positions within the brachial spinal cord (arrows).

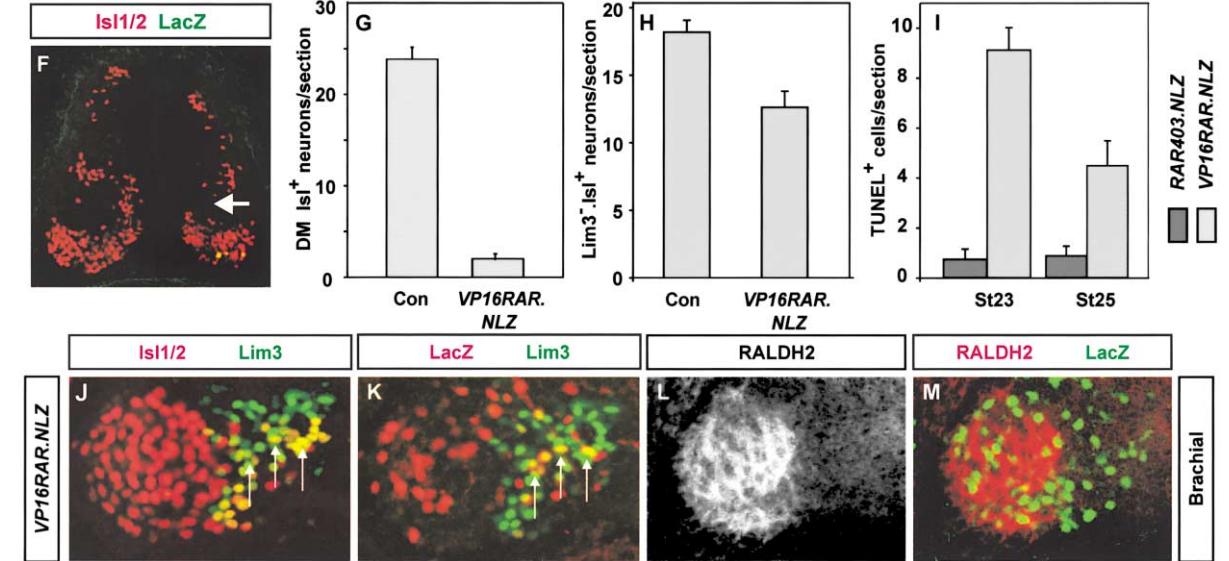
expression of *Raldh2*. This neuronal source of retinoids appears to be required for the further diversification of LMC neurons, especially for the specification of lateral LMC identity (Figure 9B; Sockanathan and Jessell, 1998). Thus, three sequential phases in the specification of brachial LMC identity appear to depend on retinoid signaling.

Several lines of evidence argue that the relevant source of retinoid signals in brachial LMC specification is the adjacent paraxial mesoderm. Retinoid synthesis in developing embryos is known to depend on the expression of three retinaldehyde dehydrogenase (*Raldh*) enzymes (Duester, 2001). At the time of motor neuron generation, *Raldh2* is expressed at high levels by brachial paraxial mesoderm but is excluded from neural cells (Niederreither et al., 1997). Moreover, neither *Raldh1* nor *Raldh3* are expressed by spinal cord cells at the time of motor neuron generation (Niederreither et al., 2002). Thus, none of the key retinaldehyde-converting enzymes is expressed in spinal progenitor cells at developmental stages relevant to brachial LMC specification. In addition, previous *in vitro* studies have revealed a critical role for retinoids provided by brachial paraxial mesoderm in interneuron patterning in the spinal cord (Pierani et al., 1999). Furthermore, the expression of *Raldh2* by motor neurons themselves appears not to be involved in the initial stages of LMC differentiation, since our data show that blockade of retinoid receptor activation in lumbar motor neurons has no effect on generic LMC identity. Together, these observations support the idea that the high level of retinoid synthesis and secretion by brachial paraxial mesoderm is responsible for the activation of retinoid receptors in postmi-

totic brachial motor neurons, thus ensuring the progression of LMC differentiation. The differing levels of *Raldh2* expression and retinoid synthesis by brachial and thoracic mesoderm is likely to contribute to the columnar respecification of motor neurons observed *in vivo* after transposition of paraxial mesoderm at a critical early developmental period (Ensini et al., 1998).

Although the activation of retinoid receptors in brachial motor neurons is required for the specification of brachial LMC identity, our findings indicate that retinoid signaling is not sufficient to direct LMC fate. The exposure of cells in thoracic spinal cord to retinoids *in vitro* (Sockanathan and Jessell, 1998) or, as shown here, to a constitutively active retinoid receptor derivative *in vivo*, does not induce LMC neurons. Thus, retinoids are likely to influence brachial LMC identity in concert with other rostrocaudal patterning factors. FGF signaling from the node and nascent paraxial mesoderm acts together with retinoids to establish a rostrocaudal pattern of Hox protein expression in the spinal cord (Liu et al., 2001; Bel-Vialar et al., 2002), raising the possibility that FGFs and retinoids cooperate in the specification of brachial LMC identity.

LMC neurons are generated at lumbar as well as at brachial levels of the spinal cord, and these two sets of motor neurons exhibit many common molecular features, including *Raldh2* expression and a common LIM HD transcription factor profile (Jessell, 2000). Strikingly, we find that blockade of retinoid receptor signaling in lumbar motor neurons fails to impair generic LMC differentiation, in marked contrast to events at brachial levels. This finding implies that retinoid signaling is involved in the initial specification of LMC identity only at brachial



**Figure 8. Constitutive Retinoid Receptor Activation in Thoracic Level Motor Neurons Perturbs the Development of CT and Lateral MMC Neurons**

(A) Number of Lim3<sup>+</sup>, Isl1<sup>+</sup> neurons at thoracic regions of electroporated (*VP16RAR.NLZ*) and controlateral (Con) sides of embryos at stage 25.

(B–F) Analysis of *VP16RAR.NLZ*-electroporated embryos at stages 23–27. (E) High-power image of the boxed area in (D); the arrow indicates LacZ<sup>+</sup> neurons that have downregulated HB9. (F) Stage 27 *VP16RAR.NLZ*-electroporated embryo shows loss of CT neurons (arrow) on the electroporated side.

(G) Decrease in dorsomedial (DM) Isl1/2<sup>+</sup> neurons in electroporated (*VP16RAR.NLZ*) versus controlateral (Con) sides of electroporated embryos ( $p = 0.0001$ ).

(H) Decrease in lateral MMC-like neurons (Lim3<sup>+</sup>, Isl1<sup>+</sup>) on the *VP16RAR.NLZ*-electroporated side of stage 32 embryos ( $p = 0.0023$ ).

(I) Number of TUNEL<sup>+</sup> LacZ<sup>+</sup> neurons at thoracic levels in stage 23 ( $p = 0.0038$  versus controls) and stage 25 ( $p = 0.0037$  versus controls) *VP16RAR.NLZ*- and *RAR403.NLZ*-electroporated embryos.

(J and K) *VP16RAR.NLZ*-electroporated neurons at stage 29 coexpress the medial MMC markers Lim3 and Isl1/2 (arrows).

(L and M) Brachial motor neurons that express *VP16RAR.NLZ* are distributed throughout the LMC and coexpress the LMC marker Raldh2.

levels of the spinal cord, again consistent with the detection of high levels of *Raldh2* expression only in brachial level paraxial mesoderm over the initial period of LMC differentiation (Figures 1A–1C; Berggren et al., 1999). The signals that impose lumbar LMC fate have not been defined but could involve factors provided by the node or caudal paraxial mesoderm (Liu et al., 2001; Lance-Jones et al., 2001).

In contrast to the selective involvement of retinoids in the specification of brachial LMC identity, our findings show that retinoid receptor activation is required for the emergence of lateral LMC identity at both brachial and lumbar levels. Thus, retinoid signals appear to be required for the specification of lateral LMC subtype identity, independent of their involvement in generic LMC specification. Our findings do not define the retinoid receptor subtypes involved in the control of generic LMC specification because *RAR403* blocks retinoid receptor

signaling mediated by both RAR-RXR heterodimers and RXR homodimers (Damm et al., 1993). Signaling through both RAR and RXR receptors has, however, been implicated previously in the specification of lateral LMC identity (Sockanathan and Jessell, 1998; Solomin et al., 1998).

The blockade of retinoid receptor activation in brachial motor neurons appears to result in a switch to thoracic columnar fates. Several independent features of CT neuronal character are evident in brachial level motor neurons after retinoid receptor blockade, notably a dorsomedial settling position and the projection of motor axons to sympathetic ganglion targets. In addition, blockade of retinoid receptor signaling results in the generation of brachial motor neurons that remain in a ventrolateral position and project axons to body wall muscles. Thus, features of both CT and lateral MMC neurons, the two columnar subtypes of motor neurons



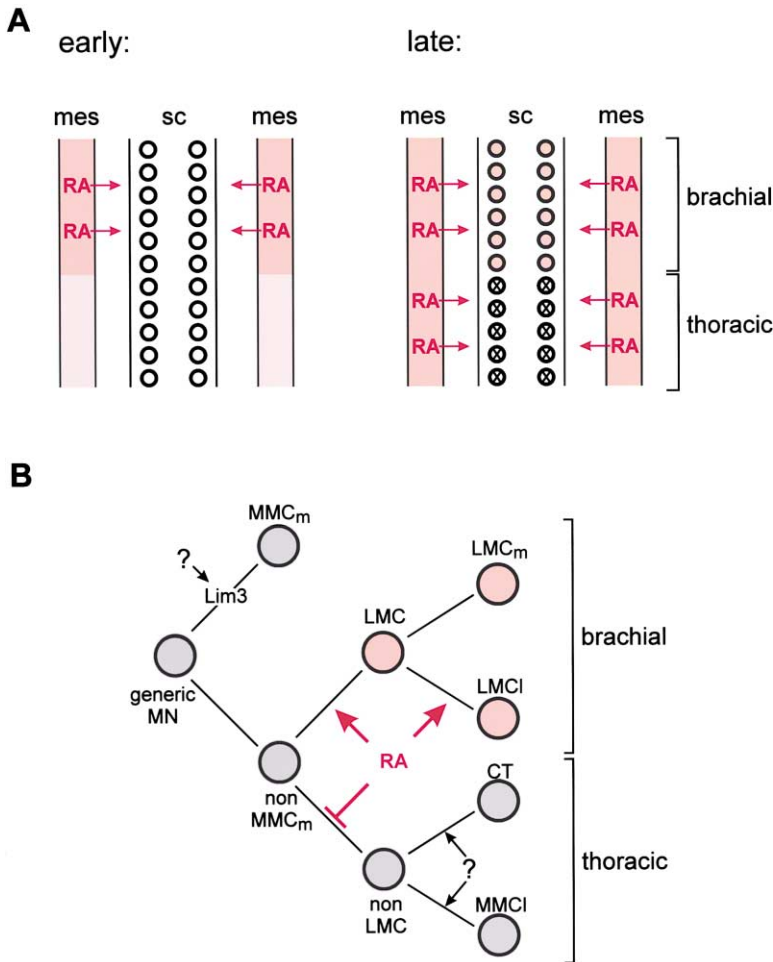


Figure 9. Postmitotic Retinoid Receptor Activation Regulates Motor Neuron Columnar Identity

(A) Dynamic patterns of retinoid signaling (RA) in brachial (forelimb) and thoracic level paraxial mesoderm (mes) over the period of motor neuron generation. Early: high-level RA signaling (red) from brachial level mesoderm and low-level RA signaling from thoracic mesoderm (light pink). Retinoid signals influence brachial levels of the spinal cord at a time when newly generated spinal motor neurons (open circles) are competent to respond to retinoids with columnar specification. Late: by the time that RA signals are provided at high levels by brachial and thoracic level paraxial mesoderm, the specification of LMC neurons is complete (indicated by red circles), and prospective CT and lateral MMC motor neurons (circles with x) may no longer be competent to respond to retinoid exposure with a respecification of columnar identity. The loss of response of thoracic level motor neurons may reflect expression of *Cyp26b*. For details, see text.

(B) Model depicting successive steps in the diversification of spinal motor neurons into distinct columnar subtypes. The initial distinction between medial MMC (MMC<sub>m</sub>) and other motor neurons is dependent on the persistence of expression of *Mnx* and *Lim3* (*Lhx3*) proteins (Sharma et al., 2000; Williams et al., 2003), although the signal that determines the duration of *Mnx* and *Lim3* expression in postmitotic motor neurons is not known. Other motor neurons (non-MMC<sub>m</sub>) undergo further diversification under the control of retinoid signals. At brachial levels, retinoid receptor activation in newly generated motor neurons is required for progression to generic LMC columnar identity, whereas eva-

sion of retinoid signals at thoracic levels is needed for progression to a non-LMC identity. Subsequently, retinoid signals provided by LMC neurons direct the acquisition of lateral LMC divisional identity (Sockanathan and Jessell, 1998). Non-LMC neurons subsequently diverge to generate both CT and lateral MMC neurons, although the factors that control this decision remain unclear. MMC<sub>m</sub> neurons are generated at all axial levels of the spinal cord but are not shown in this model, since they appear uninfluenced by retinoid exposure. For further details, see text.

normally generated at thoracic levels, are acquired by brachial motor neurons under conditions of retinoid receptor blockade. The steps that direct the divergence of CT and lateral MMC identity remain to be defined, but our findings suggest that this process does not involve retinoid signaling (Figure 9B).

#### Retinoid Signaling and the Differentiation of Thoracic Motor Neurons

The emergence of CT and lateral MMC neuronal character after blockade of retinoid receptor activation in brachial motor neurons raises the issue of the fate of CT and lateral MMC neurons at thoracic levels under conditions of retinoid receptor activation (Figure 9B). We find that expression of *VP16RAR* in thoracic level motor neurons perturbs the differentiation of CT and lateral MMC neurons, causing thoracic motor neurons to undergo apoptotic death. The actions of *VP16RAR* appear to be specific, since the differentiation of medial MMC neurons and LMC neurons is not perturbed by *VP16RAR* expression. In addition, expression of *VP16RAR* in neu-

ral progenitor cells, in the context of FGF signaling, actually promotes motor neuron generation (Novitsch et al., 2003 [this issue of *Neuron*]). These findings, therefore, suggest that the normal progression of newly generated thoracic level motor neurons to CT and lateral MMC fates is perturbed by high-level retinoid signaling.

Previous studies have shown that early expression of *Raldh2* in vivo or retinoid exposure in vitro induces a lateral LMC-like identity in thoracic motor neurons (Sockanathan and Jessell, 1998). In these prior experiments, thoracic level progenitor cells were exposed to retinoids (Sockanathan and Jessell, 1998), whereas in the present studies retinoid receptor activation is restricted to postmitotic neurons. A likely explanation for these findings is that the specification of lateral LMC identity in thoracic level motor neurons requires an early phase of retinoid signaling in progenitor cells, as well as a later phase of signaling in postmitotic motor neurons.

How do CT and lateral MMC neurons escape high-level retinoid signaling? One contributing factor is likely to be the low level of *Raldh2* expression in thoracic level mesoderm (Figures 1A–1C and 9A; Berggren et al., 1999;



Swindell et al., 1999). As a consequence, the neural tube at thoracic levels appears to be exposed to only a low level of retinoid signaling over the time period of motor neuron columnar specification (Solomin et al., 1998). And by the time that *Raldh-2* expression in the paraxial mesoderm flanking thoracic levels of the spinal cord increases (Berggren et al., 1999), thoracic motor neurons may have lost their sensitivity to the disruptive effects of retinoid receptor activation (Figure 9A; see also Liu et al., 2001). One further mechanism for evasion of retinoid signaling may involve an enhanced capacity of thoracic motor neurons to degrade active retinoids. Motor neurons at thoracic but not brachial levels express *Cyp26b*, a p450-related enzyme that converts morphogenetically active retinoids into less active oxidative metabolites (Abu-Abed et al., 2002; J. Dasen and T.M.J., unpublished observations). Thus, the initial specification of CT and lateral MMC neuronal identities may result in *Cyp26b* expression and elevated retinoid degradative capacity, providing secondary protection against later exposure to retinoid signals.

#### Postmitotic Retinoid Signaling and the Emergence of Motor Neuron Columnar Identity

The manipulations of retinoid receptor signaling analyzed in these studies involve a promoter that directs transgene expression to postmitotic neurons (Arber et al., 1999; William et al., 2003). One implication of our findings, then, is that the subtype identity of spinal motor neurons is mutable after cell cycle exit. This conclusion is supported by other studies of motor neuron differentiation. Altering the profile of expression of *Mnx* class HD proteins or reducing *Isl1* and *HB9* HD protein dosage in postmitotic motor neurons promotes a visceral to somatic switch in motor neuron subtype identity (William et al., 2003; J.P. Thaler et al., submitted). In contrast, maintained expression of *Lhx3* (*Lim3*) in postmitotic motor neurons promotes medial MMC identity and represses CT and LMC fate (Sharma et al., 2000; William et al., 2003). Aspects of the pool identity of LMC neurons, revealed by ETS protein expression, are acquired at an even later stage, in response to inductive signals from the limb that include Glial Derived Neurotrophic Factor (GDNF) (Lin et al., 1998; Haase et al., 2002; Livet et al., 2002).

Finally, our findings provide further support for the idea that neuronal patterning in the spinal cord involves the reiterative use of signaling factors by nonneural and neural cell groups (Jessell, 2000). Retinoid signaling from the paraxial mesoderm, and later from motor neurons themselves, helps to establish the fine-grained transcriptional identity of motor neuron subtypes that is essential for the neural control of limb motility and locomotion. Since local sources of retinoid synthesis are detected in many regions of the developing brain (Toresson et al., 1999; Denisenko-Nehrbass et al., 2000; Wagner et al., 2002), the action of retinoid signals on postmitotic neurons could well have a widespread role in directing the identity of neuronal subtypes in the CNS.

#### Experimental Procedures

All findings are representative of results obtained from at least eight electroporated embryos.

#### In Situ Hybridization and Immunohistochemistry

In situ hybridization was performed as in Tsuchida et al. (1994). *Shh* probes are described in William et al. (2003) and Ericson et al. (1995), and images were collected on a Zeiss Axioskop microscope. Immunohistochemistry was performed as in Tsuchida et al. (1994). Most antibodies used are described in Sockanathan and Jessell (1998) with the following additions: anti-Nkx6.1 and anti-HB9 antibodies as in Novitsch et al. (2001), goat anti-HRP (Jackson Laboratories), rabbit anti-GFP (Molecular Probes), and goat anti-LacZ (Arnel).

#### Expression Constructs and In Ovo Electroporation

Human RAR403 and VP16RAR fusions were subcloned under the control of a 9 kb 5' fragment of the mouse *HB9* promoter (Arber et al., 1999) and cloned into bicistronic constructs incorporating reporter cassettes (Arber et al., 1999; Novitsch et al., 2003 [this issue of *Neuron*]). In ovo electroporation was carried out as described in Novitsch et al. (2001), and embryos were analyzed from stages 23 to 32. Images were collected on a BioRad MRC1024 confocal microscope.

#### Retrograde Labeling of Motor Neurons

Retrograde labeling was carried out using FITC-dextran (2 mg/ml) from ventral root fills or HRP (20%; Boehringer)/Isolecithin (1%) injection into target tissues, as described in Kania et al. (2000). Labeling was typically carried out for 3 hr for ventral root fills, and for 5 to 6 hr for muscle and sympathetic ganglion labeling. HRP injections were carried out primarily on embryos that had been electroporated with eGFP, rather than LacZ.

#### Statistical Analyses and Motor Neuron Quantitation

Quantitation of motor neuron number was typically obtained from 10–20 confocal micrographs obtained from five different experimental embryos. Graphical constructions and statistical analyses were carried out using Sigmaplot 8.0. To obtain the results graphed in Figure 4, the ventral quadrant of the spinal cord was divided into a grid consisting of six divisions along the mediolateral axis and four divisions along the dorsoventral axis, where position 1-1 corresponds to the most dorsomedial position and position 6-4 corresponds to the most ventrolateral position. LacZ<sup>+</sup> motor neurons in each bin were expressed as a percentage of the total number of neurons.

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